# The effectiveness of a dietary direct-fed microbial and mannan oligosaccharide on ultrastructural changes of intestinal mucosa of turkey poults infected with *Salmonella* and *Campylobacter*

Shaban Rahimi,[*] Sophia Kathariou,[†] Oscar Fletcher,[‡] and Jesse L. Grimes[§,1]

[*]*Department of Poultry Science, Faculty of Agriculture, Tarbiat Modares University, 14115-336 Tehran, Iran;* [†]*Department of Food Bioprocessing and Nutrition Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27695-7608;* [‡]*Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27695-7608; and* [§]*Prestage Department of Poultry Science, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, NC 27695-7608*

**ABSTRACT** *Salmonella* and *Campylobacter* are considered major public health burdens worldwide, and poultry are known to be one of the main reservoirs for these zoonotic pathogens. This study was conducted to evaluate the effect of a commercial probiotic or direct-fed microbial (**DFM**) Calsporin (**CSP**), and prebiotic or mannan oligosaccharide (**MOS**) (**IMW50)** on ultrastructural changes and the villous integrity of intestinal mucosa in turkey poults challenged with *Salmonella* and *Campylobacter*. A 21-day battery cage study was conducted using 4 dietary treatments including a basal diet (corn and soybean–based) nonsupplemented and uninfected as a negative control (NC); basal diet supplemented with 0.05% DFM (CSP); basal diet supplemented with 0.05% MOS (IMW50); and basal diet supplemented with 0.05% mixture of DFM and MOS at equal proportions. Female large white turkey poults aged 336 days were obtained from a local commercial hatchery and randomly distributed in electrically heated battery cages with 12 treatments of 4 replicates per treatment containing 7 poults per pen. The first 16 pens were not infected with bacteria, poults in pens 17-32 were orally challenged at day 7 with $10^5$ cfu *Salmonella* Heidelberg, and the poults in pens 33-48 were orally challenged at day 7 with $10^5$ cfu *Campylobacter jejuni*. Feed and water were provided ad libitum throughout the study. At day 21, ileal tissue samples from 1 bird per cage were collected for intestinal integrity and ultrastructural examination by scanning and electron microscopy. DFM and MOS supplementation was effective in both challenged and nonchallenged (not infected with *Salmonella* and *Campylobacter*) birds. Goblet cells and mucus were increased, with the presence of large numbers of segmented filamentous bacteria in DFM- and MOS-supplemented groups compared with birds in control treatments. The number and size of villi were reduced in poults exposed to *Salmonella* and *Campylobacter*. Results show that CSP and IMW50 provide protection of ileal mucosal integrity in poults exposed to *Salmonella* or *Campylobacter*.

**Key words:** DFM, MOS, *Campylobacter*, *Salmonella*, Turkey poult

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## INTRODUCTION

*Salmonella* and *Campylobacter* are important human foodborne pathogens, commonly associated with poultry and poultry products (CDC, 2006; Johnson et al., 2017). These are the leading cause of foodborne infections in both developed and developing countries worldwide (Carter et al., 2009). In the United States, *Campylobacter* and *Salmonella* caused the most reported bacterial foodborne illnesses in 2016, according to preliminary data published in CDC's *Morbidity and Mortality Weekly Report*. CDC's Foodborne Diseases Active Surveillance Network (FoodNet) collects data on 15 percent of the United States population. FoodNet sites alone reported 24,029 foodborne infections, 5,512 hospitalizations, and 98 deaths in 2016. The numbers of reported illnesses by germs are as follows: *Campylobacter* (8,547), *Salmonella* (8,172), *Shigella* (2,913), Shiga toxin–producing



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[1]Corresponding author: jesse_grimes@ncsu.edu

*Escherichia coli* (1,845), *Cryptosporidium* (1,816), *Yersinia* (302), *Vibrio* (252), *Listeria* (127), and *Cyclospora* (55) (CDC, 2016). In 2016, USDA's Food Safety and Inspection Service (FSIS) finalized new performance standards for reducing harmful bacteria in chicken parts and ground poultry. FSIS expects these actions could prevent as many as 50,000 illnesses each year caused by Salmonella and Campylobacter in chicken and turkey products. Salmonella Typhimurium infections, often linked to beef and poultry, decreased 18 percent in 2016 compared with the average for 2013-2015. The continuing decreases in Salmonella Typhimurium may be due to regulatory action to reduce Salmonella contamination in poultry and vaccination of chicken flocks by producers. Reported Yersinia, Cryptosporidium, and Shiga toxin-producing E.coli infections increased. These increases are likely due to newly available rapid tests that make infections easier to diagnose, rather than to a true increase in illness.

In the European Union, it is estimated that there are approximately 9 million cases of human campylobacteriosis per year with an annual cost of about 2.4 billion € (EFSA, 2011). It is estimated that the global burden of 95 million illnesses, 21,000 deaths, and 2.1 million disability-adjusted life years occurred because of campylobacteriosis in 2010 (Havelaar et al., 2015). In the United States, treatment of acute disease and postinfectious disorders associated with *Campylobacter* infection cost approximately $1.7 billion USD annually (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5362611/,Maue et al., 2014).

Of the 17 established *Campylobacter* species, the most important one associated with human disease is *Campylobacter jejuni*, a leading cause of diarrheal disease worldwide with 400-500 million laboratory-confirmed cases each year (Apajalahati et al., 2004, Ruiz-Palacios, 2007).

Antibiotic growth promoters are known to reduce *Salmonella* colonization in the gastrointestinal (**GI**) tract of poultry (Dibner and Richards, 2005), but the worldwide use of antibiotics in animal husbandry has led to increased antibiotic-resistant bacteria (Barnza, 2002; Bywater, 2005). Use of functional feeds such as probiotics and prebiotics has been enhanced for industrial health and to obtain safe, reliable, and high-quality animal production without any antibiotics (Torres-Rodriguez et al., 2005; Willis et al., 2007; Grimes et al., 2008).

Probiotics as live bacteria (Fuller, 1991) and prebiotics (which encourage the growth of beneficial bacteria) could be used as antibiotic alternatives in animal feed. Probiotics, which are live, nonpathogenic bacteria, demonstrated direct-fed microbials (**DFM**) that contribute to improved health and balance of the GI tract (Apajalahti, 2004). Prebiotics are defined as food ingredients that selectively stimulate the growth and activity of beneficial microorganisms such as *Bifidobacteria* and *Lactobacillus* in the gut and reduce colonization of pathogenic microorganisms and thus benefit health (Cummings and MacFarlane, 2002).

**Table 1.** Composition of the ration for rearing turkey poults to 21 D.

| Ingredient | % |
|---|---|
| Item | |
| Corn | 43.40 |
| Soybean meal | 46.00 |
| Poultry fat | 4.00 |
| Dicalcium phosphate | 3.80 |
| limestone | 1.00 |
| Lysine | 0.40 |
| Salt | 0.45 |
| DL-Methionine | 0.25 |
| Choline chloride | 0.20 |
| Minerals[1] | 0.20 |
| Vitamins[2] | 0.20 |
| Selenium premix[3] | 0.10 |
| CSP, IMW50, (CSP + IMW50)[4] | 0.50 |
| Calculated nutrient content | |
| Crude protein | 27.00 |
| ME (kcal/kg) | 2,925.00 |
| Fat (%) | 6.10 |
| Methionine (%) | 0.65 |
| TSAA (%) | 1.04 |
| Lysine (%) | 1.81 |
| Calcium (%) | 1.34 |
| Available P (%) | 0.73 |

Abbreviations: DFM, direct-fed microbial; MOS, mannan oligosaccharide.

[1]Minerals mix supplied the following per kilogram of diet: 120 mg of Zn as $ZnSO_4$, $H_2O$; 120 mg of Mn as $MnSO_4$ $H_2O$; 80 mg of Fe as $FeSO_4.H_2O$; 10 mg of Cu as $CuSO_4$; 2.5 mg of I as $Cu(IO_3)_2$; 1.0 mg of Co as $CoSO_4$.

[2]Vitamin mix supplied the following per kilogram of diet when added at 0.2%: vitamin A, 6,600 IU; vitamin $D_3$, 2000 ICU; vitamin E, 33 IU; vitamin $B_{12}$, 19.8 µg; riboflavin, 6.6 mg; niacin, 55 mg; D-pantothenate, 11 mg menadione, 2 mg; folic acid, 1.1 mg; thiamine, 2 mg; pyridoxine, 4 mg; D-biotin, 126 µg; ethoxyquin, 50 mg.

[3]Slenium premix supplied 0.21 mg Se, as $Na_2SeO_3$.

[4]DFM Calsporin (CSP), MOS (IMW50) and mixture of DFM and MOS (CSP + IMW50) (QIT, Inc, Elign, IL) provided at 500 g/ton of feed in different treatments based on the experiment design.

Gut microflora affect poultry health through their effects on gut morphology, nutrition, pathogenesis of intestinal diseases, and immune responses. The microbial flora are also believed to protect against colonization of the intestines by pathogens and to stimulate immune responses (Mead, 2000). Intestinal bacteria are primarily responsible for degrading the copious amounts of mucus

**Table 2.** Effect of [1]direct-fed microbial (DFM) and [2]prebiotic (MOS) on intestinal villi density of 21-day-old [3]poults (mean ± SD).

| Challenge<br>Feed | Non-challenged | *Salmonella* challenged | *Campylobacter* challenged |
|---|---|---|---|
| C | 24 ± 7.1 | 23 ± 3.6 | 20 ± 4.9 |
| DFM | 28 ± 2.2 | 28 ± 7.3 | 25 ± 3.8 |
| MOS | 26 ± 6.1 | 28 ± 5.1 | 23 ± 6.8 |
| DFM + MOS | 26 ± 3.4 | 27 ± 6.6 | 26 ± 6.8 |
| SEM | 2.57 | 2.92 | 2.87 |
| P-Value | 0.75 | 0.59 | 0.44 |

*Statistical analysis.* All the data were analyzed by one-way ANOVA (SAS, 1998) using a completely randomized design with 4 feed treatments (control, DFM, MOS, and DFM + MOS) and 4 replicates of each. Differences between treatment means were considered significant at $P \leq 0.05$.

Abbreviation: MOS, mannan oligosaccharide.

[1]Calsporin (DFM) and [2]IMW (MOS) provided at 0 .5 g/kg feed.

[3]Poults were gavaged at 7 D with *Salmonella* Heidelberg $10^5$ cfu and *Campylobacter jejuni* $10^5$ cfu.

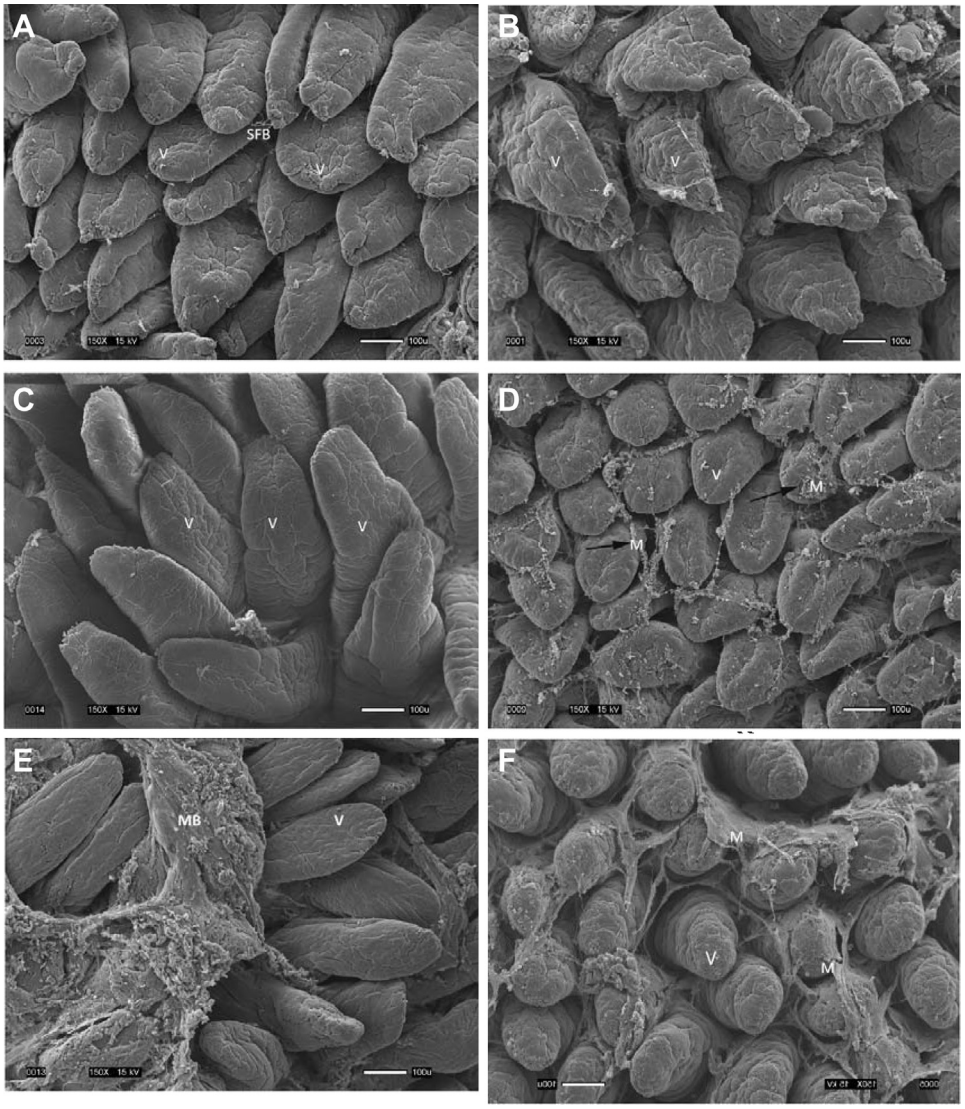


**Figure 1.** SEM images of the ileum from nonchallenged poults. The poults supplemented with DFM and MOS show intact villous architecture. It seems that the number and height of villi (V) in DFM- (A), MOS- (B), and mixture of DFM and MOS– (C) supplemented birds are higher than those in the control group (not supplemented with DFM or MOS) (D). The number of villi (V) and amount of mucus secretion (M) in DFM- (E) and mixture of DFM and MOS– (F) fed birds were higher than in birds in the control group (not supplemented with DFM or MOS) (D). (E) Tongue-shaped villi (V) and high secretion of a mucus blanket layer (MB) covering enterocytes. DFM, direct-fed microbial; MOS, mannan oligosaccharide; SEM, scanning electron microscopy.

produced by goblet cells in the intestine (Meslin et al., 1999; Falk et al., 2000). However, many factors, such as diet (Knarreborg et al., 2002), age (van der Wielen et al., 2002; Zhu et al., 2002), and infection by pathogenic organisms (Kimura et al., 1976) can affect the composition of the avian bacterial community. The segmented filamentous bacteria (**SFB**) from gut microflora are noncultivable, commensal bacteria that strongly adhere to the epithelial cells of the ileum and of the Peyer's patches where they inhibit adhering pathogens. The commensal bacteria participate in protection against intestinal pathogens. They occupy important ecological niches and compete with ingested pathogens for nutrients; they strengthen the epithelial barrier and occupy epithelial cell adhesion receptors, decreasing the invasive capacity of pathogens; they produce ligands that activate pattern recognition receptors, for example, Toll-like receptors, resulting in secretion of host bactericidal substances, such as antimicrobial peptides, and in immunostimulatory signals that help recruit B and T cells in the lamina propria, organize secondary lymphoid structures, and induce IgA secretion into the lumen; and they secrete molecules, such as lactic acid, that inhibit the growth of competing microorganisms. Thus, the intestinal microbiota represent an integral and vital part of the host organism and are therefore referred to as the "microbial organ" (Ivanov and Littman, 2010). Colonization of SFB induces IgA-producing cells in the lamina propria of the small intestine and αβ-T-cell receptor–bearing intraepithelial lymphocytes (Umesaki et al., 1995).

Calsporin **(CSP,** Calpis Co. Ltd., Tokyo, Japan) is a commercial DFM that contains the naturally occurring *Bacillus subtilis* strain C-1302. *B. subtilis* is thought to have a weak ability to cause disease in humans unless the number of bacteria a person consumes is very high

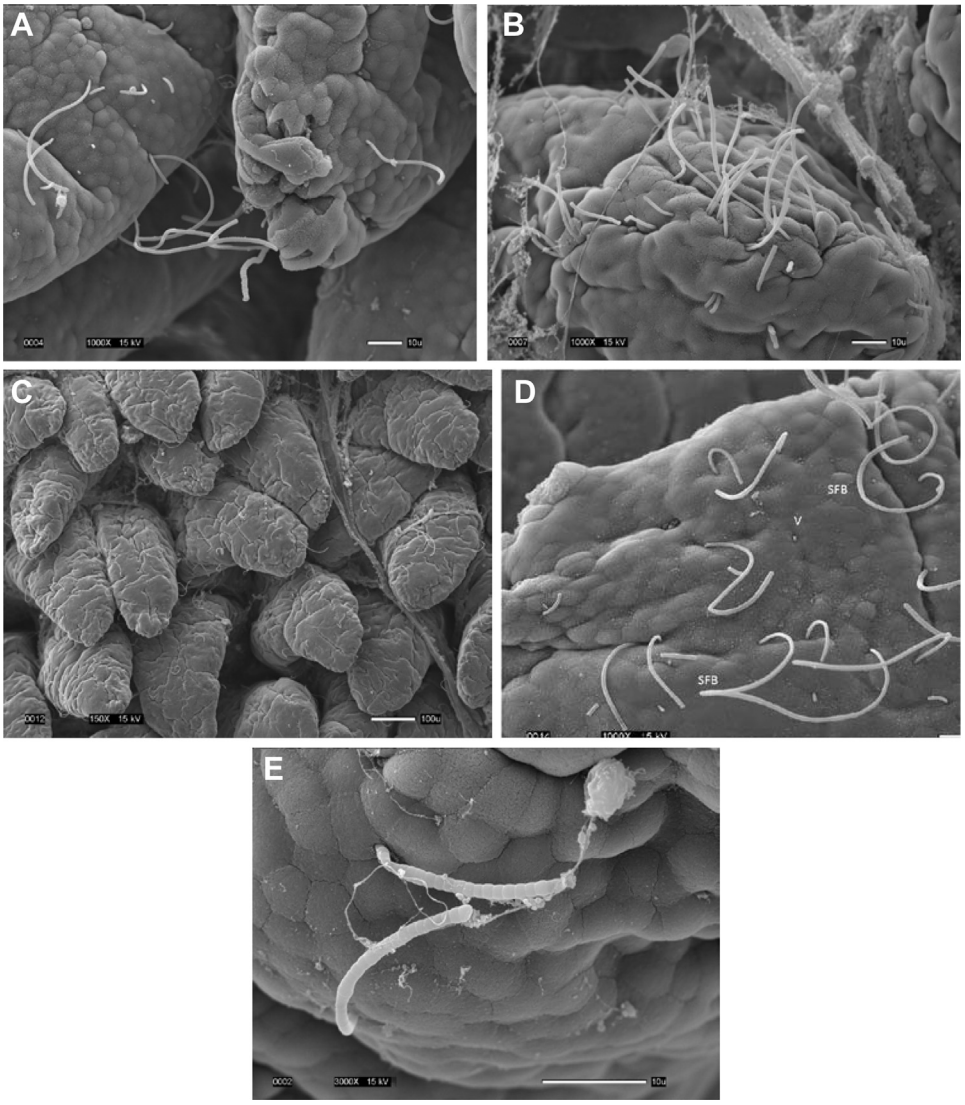


**Figure 2.** SEM images of SFB in the ileum of nonchallenged poults. The number of SFB in DFM- (A, B) and mixture of DFM and MOS– (C, D) fed birds was higher than in the control group (E). SFB show a characteristic long filamentous morphology. SFB heavily colonized on tips and sides of absorptive villi (V), providing the intestinal surface with a tufted appearance (B). Filamentous organisms penetrate the intestinal cell either at their surface or between enterocytes. Two morphotypes can be distinguished into 1) smooth filaments and 2) filaments with a beaded appearance (E). DFM, direct-fed microbial; MOS, mannan oligosaccharide; SEM, scanning electron microscopy; SFB, segmented filamentous bacteria.

or the immune status of the person is very low. Probably owing to the possibility of any virulence, no *Bacillus subtilis* strain has GRAS status as of March, 2015 in the United States. While some sites claim theirs does, this does not to appear to have been substantiated. Some enzymes produced from *B. subtilis* have GRAS status, but the bacterium itself does not (Anonymous, 2015).

The genus *Bacillus* comprises a diverse collection of aerobic endospore-forming bacteria whose spores are extremely resistant to external physical and chemical insults and in part determines their exceptional longevity in the environment (Henriques et al., 2000; Nicholson et al., 2000). Nevertheless, it is anticipated that the competitive exclusion of pathogens by *Bacillus* probiotics results from one or more modes of action, including immune exclusion, competition of adhesion sites, and production of antimicrobial agents, such as bacteriocins (Patterson and Burkholder, 2003).

Prebiotic mannan oligosaccharide (**MOS**) is derived from mannans on yeast cell surfaces. It is not used as a substrate in microbial fermentation but still exerts a significant growth-promoting effect by enhancing the animal's resistance to enteric pathogens. Based on the literature, MOS enhances an animal's resistance to enteric disease and promotes growth by the following means: 1) inhibits colonization of enteric pathogens by blocking bacterial adhesion to the gut lining; 2) enhances immunity; 3) modifies microflora fermentation to favor nutrient availability for the host; 4) enhances the brush border mucin barrier; and 5) reduces enterocyte turnover rate (Ferket, 2011). Ferket (2002) reported turkey poults fed dietary supplementation of MOS had a significant effect on intestinal villi morphology of turkey poults in comparison with those fed nonmedicated control or virginiamycin-supplemented diets. Dietary MOS supplementation (Ferket, 2002) and dietary DFM

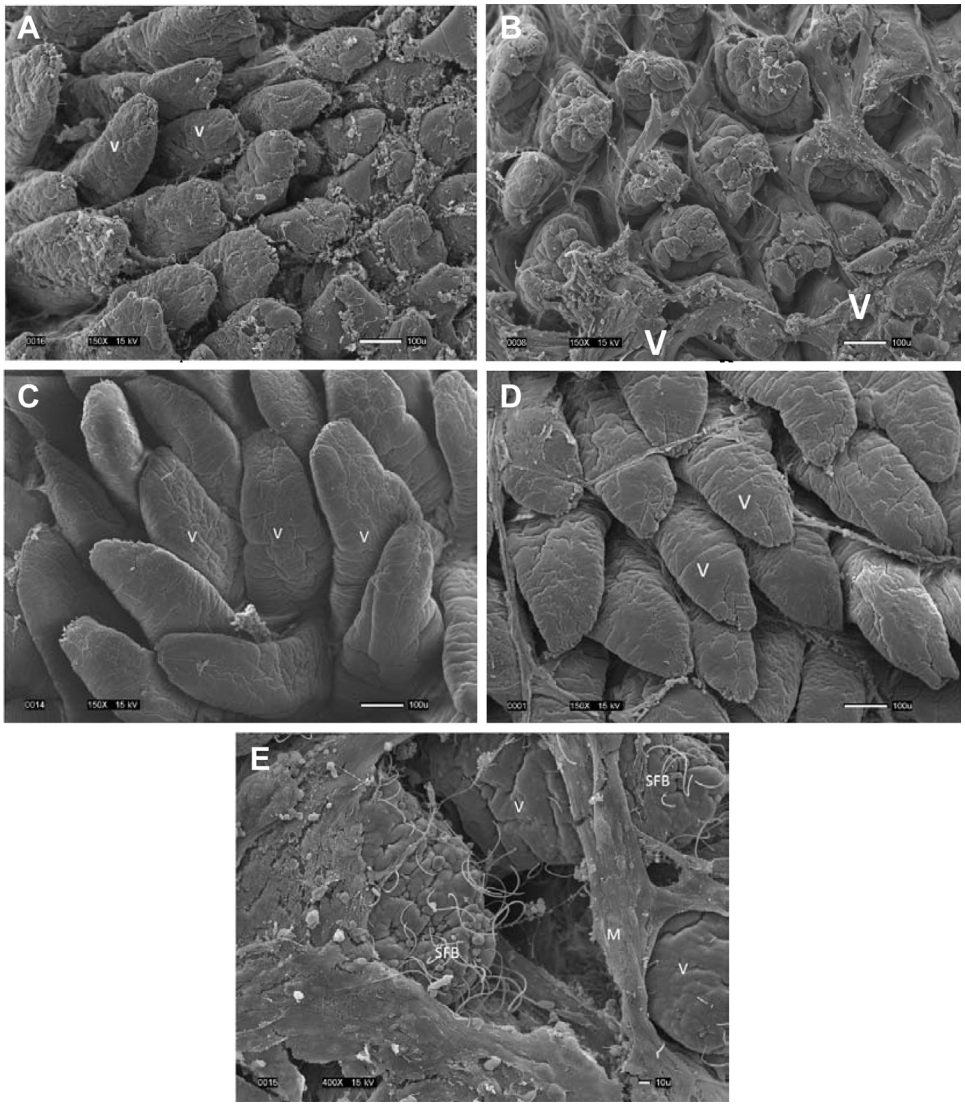


**Figure 3.** SEM images of the ileum in *Salmonella*-challenged poults. The number and height of villi in DFM- (A), MOS- (B), and mixture of DFM and MOS– (C) supplemented birds were higher than in the control bird (challenged, not DFM and MOS supplemented) (D). Amount of mucus secretion (M) was higher in DFM- (A, E) and MOS- (B) fed birds than in the control bird (challenged, not DFM and MOS supplemented) (D). (E) High secretion of mucus (M) covering the structural detail of the intestinal surface of the ileal villi. DFM, direct-fed microbial; MOS, mannan oligosaccharide; SEM, scanning electron microscopy.

supplementation (Rahimi et al., 2009) significantly increased villi height-to-crypt depth ratio and increased the number and size of the goblet cells relative to villus height. The mucus gel layer coating the surface of the intestinal epithelium is the first barrier to enteric infection against enteric pathogens.

The prevalence of bacteria in different parts of the GI tract appears to be dependent on several factors, such as pH, peristalsis, redox potential, bacterial adhesion, bacterial cooperation, mucin secretion, nutrient availability, diet, and bacterial antagonism. Because of the low pH of the stomach and the relatively swift peristalsis through the stomach and the small bowel, the stomach and the upper two-thirds of the small intestine (duodenum and jejunum) contain only low numbers of microorganisms, which range from $10^3$ to $10^4$ bacteria/mL of the gastric or intestinal contents, mainly acid-tolerant lactobacilli and streptococci. In the distal small intestine (ileum), the microflora begin to resemble those of the colon, with around $10^7$-$10^8$ bacteria/mL of the intestinal contents. With decreased peristalsis, acidity, and lower oxidation–reduction potentials, the ileum maintains a more diverse microflora and a higher bacterial population (Hao and Lee, 2004).

The objective of this study was to investigate the influence of a DFM (CSP) and a MOS derived from the *Saccharomyces cerevisiae* (IMW50, Quality Technology International, Inc., Elgin, IL) on the integrity of the enteric mucosa and ultrastructural changes in the ileum of turkey poults infected with *Salmonella* and *Campylobacter*.

## MATERIALS AND METHODS

Bird handling and experimental design were performed as reported by Rahimi et al., 2019 (In Press).

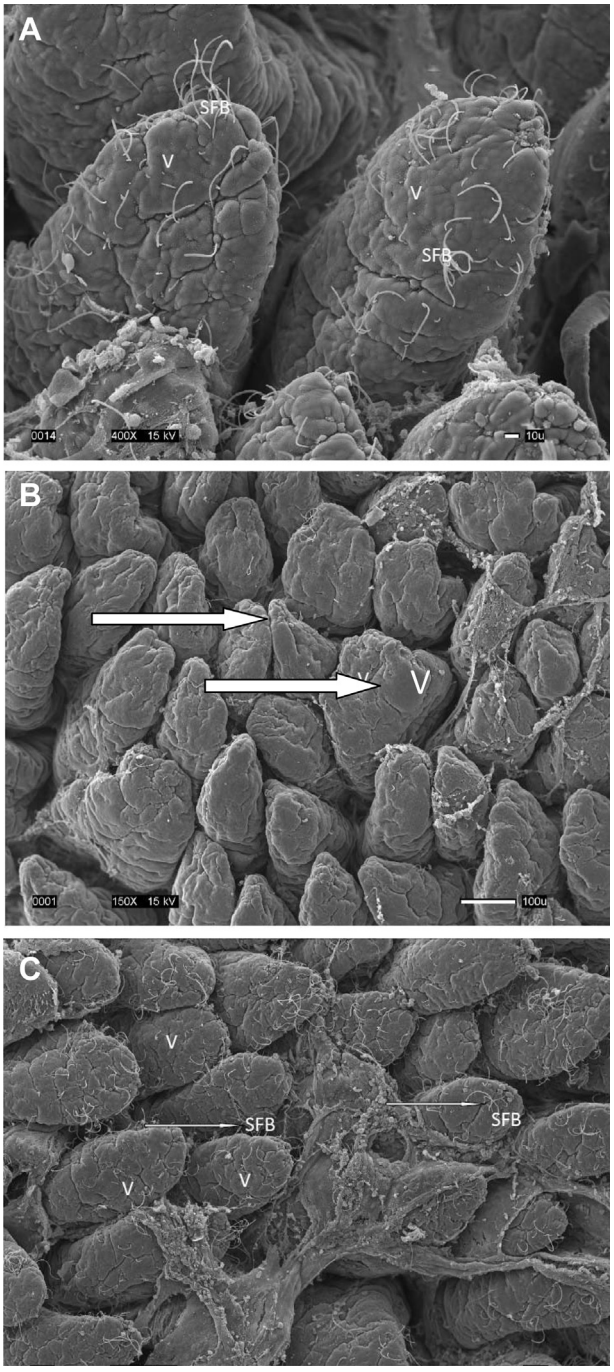


**Figure 4.** SEM images of SFB in the ileum of *Salmonella*-challenged poults. SFB colonization is shown on the ileal villi. The number of SFB in DFM-treated birds (thin arrow) (A, C) was higher than in the control group (B). Filaments are attached to epithelial cells without any signs of inflammation. (B) Slight destruction of villus tips in *Salmonella*-challenged and not DFM and MOS–supplemented control poults (thick arrow). DFM, direct-fed microbial; MOS, mannan oligosaccharide; SEM, scanning electron microscopy; SFB, segmented filamentous bacteria.

All bird handling procedures were approved by the NC State University Institutional Animal Care and Use Committee. The 21-D study was conducted using 336 one-day-old female large white turkey poults (85 × 700, Nicolas Select, Aviagen Turkey Breeding Farms, Lewisburg, WV) in a battery cage system at the Talley Turkey Education Unit, North Carolina State University. The poults received 1 of 4 dietary treatments: T1) negative, unsupplemented control (NC) (corn and soybean–based); T2) DFM (CSP; 0.05% in feed); T3) MOS (IMW50; 0.05% in feed); and T4) 0.05% mixture of DFM and MOS at equal proportions in basal diet feed, for a period of 21 D using a completely randomized design. The corn and soybean–based crumbled diet was formulated as shown in Table 1. Both the DFM and MOS were obtained from Quality Technology International, Inc. (Elgin, IL). The DFM and MOS feeds were mixed after all nontreated feed was mixed and bagged.

A total of 48 replicate pens contained 7 poults each. Poults were weighed individually, wing banded, and randomly segregated into treatment groups. The experimental design included 3 challenge groups, each with the 4 dietary treatments containing 4 replicates per treatment and 7 birds in each replicate. One-third of the birds (pens 1-16) were not infected with either *Salmonella* or *Campylobacter*. All poults in pens 17-32 were orally challenged with $10^5$ cfu *Salmonella* Heidelberg, and all poults in pens 33-48 were orally gavaged with $10^5$ cfu C*ampylobacter jejuni* 11601MD (Dutta et al., 2016) at 7 days of age. All tasks were performed with control birds first and then with bacteria-challenged birds. Biosafety level 2 practices were applied during the experiment, and all work with live bacteria was performed under a biosafety hood.

## Statistical Analysis

All the data were analyzed by two-way ANOVA (JMP, version 8.0, SAS Institute, 1998) within a completely randomized design in a 3 (nonchallenged control, *Salmonella*-challenged, and *C. jejuni* challenged) × 4 (control, DFM, MOS, and DFM + MOS) factorial arrangement. Differences between treatment means were considered significant at $P \leq 0.05$. In this experiment, we compared 4 electron micrographs of villus density from each dietary treatment including control, DFM, MOS, and mixture of DFM and MOS at 150× magnification.

## Tissue Sampling for Electron Microscopy

At 21 D, 2 bird per pen was randomly selected and humanely sacrificed by cervical dislocation, necropsied, and sections of ileum obtained to evaluate the integrity of the intestinal mucosa. Sample for scanning electron microscopy (**SEM**) and transmission electron microscopy (**TEM**) were prepared as follows:

## Scanning Electron Microscopy

Immediately after decapitation, the ileum samples from 1 bird per pen of each treatment group were taken approximately 1 cm below Meckel's diverticulum and flushed with 0.1 *M* phosphate-buffered saline (pH 7.4). The samples were prepared through several gentle washing steps, then fixed in 3% buffered glutaraldehyde (in 100 m*M* phosphate buffer, pH 7.4), and further processed in the Center for Electron Microscopy at North Carolina State University. Samples were washed 3 times in the buffer, postfixed in buffered 2% osmium tetroxide

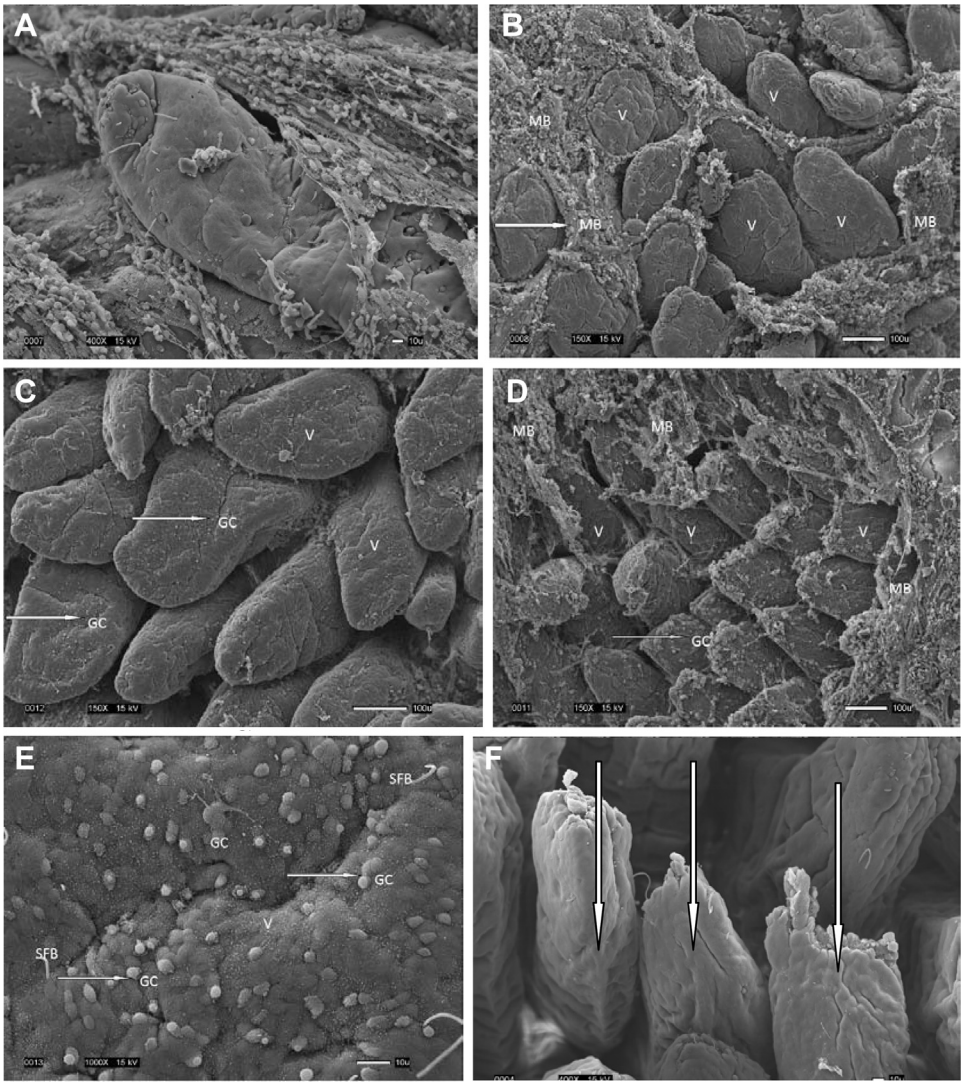


**Figure 5.** SEM images of the ileum in *Campylobacter*-challenged poults. Amount of mucus secretion (mucus blanket, MB) on villus tips (V) in DFM-supplemented birds (A), MOS-supplemented birds (B) and numbers of goblet cells (GC) in the mixture of DFM and MOS– (C, E) supplemented birds were higher than in the control (challenged, not DFM and MOS supplemented) group (F). (F) Destruction and atrophy of villus tips in *Campylobacter*-challenged but not DFM and MOS–supplemented control birds. Villi (V) have denuded sides and tips (thick arrow) with exposed lamina propria (LP). Note the loss of morphology of villi and degraded villus structure. (D) and (E) show high densities of goblet cells (thin arrows) on ileal villi. DFM, direct-fed microbial; MOS, mannan oligosaccharide; SEM, scanning electron microscopy.

(in 100 m*M* phosphate buffer, pH 7.4) for 2 h, and washed again in phosphate buffer. The samples were dehydrated in a graded ethanol series, dried in a liquid $CO_2$ critical point dryer (Samdri-795, Tousimis, Rockville, MD), secured to stubs with silver paint, and sputter coated with approximately 50-nm gold or palladium (Anatech Hummer 6.2, Anatech USA, Hayward, CA). Observation was made at 15 KV using a JEOL JSM-5900LV scanning electron microscope (JEOL U.S.A., Peabody, MA). Electron micrographs were taken from different areas of the samples for estimating morphology of villi, amount of mucus, density of goblet cells (**GC**), and bacterial colonization using a JEOL digital scan generator.

## *Transmission Electron Microscopy*

The samples of the ileum were washed in 100 m*M* phosphate buffer (pH 7.4), fixed in 3% buffered glutaraldehyde, washed 3 times in the buffer, postfixed in buffered 2% osmium tetroxide (in 100 m*M* phosphate buffer) for 2 h, and washed again in phosphate buffer. The samples were dehydrated in serial ethanol solutions (30, 50, 70, 95, and 100%) and then infiltrated in 24 h changes of 1:1 ethanol:resin (Spurr's, Electron Microscopy Sciences, Hatfield, PA), 1:3 ethanol:resin, and 3 changes of 100% resin, for 24 h each change. Vacuum infiltration was used in the three 100% resin steps. Samples were embedded in fresh resin in flat embedding molds for orientation purposes and polymerized at 70°C overnight. Blocks were trimmed to remove excess resin. Ultrathin sections were cut at approximately 75 nm using an LKB Nova ultramicrotome (Leica Microsystems, Buffalo Grove, IL) fitted with a diamond knife (Diatome), collected on copper grids, and stained with uranyl acetate and Reynold's lead citrate. Electron micrographs (4,000× and 10,000×) of intestinal mucosal cells and microvilli were taken using the a JEOL JEM-

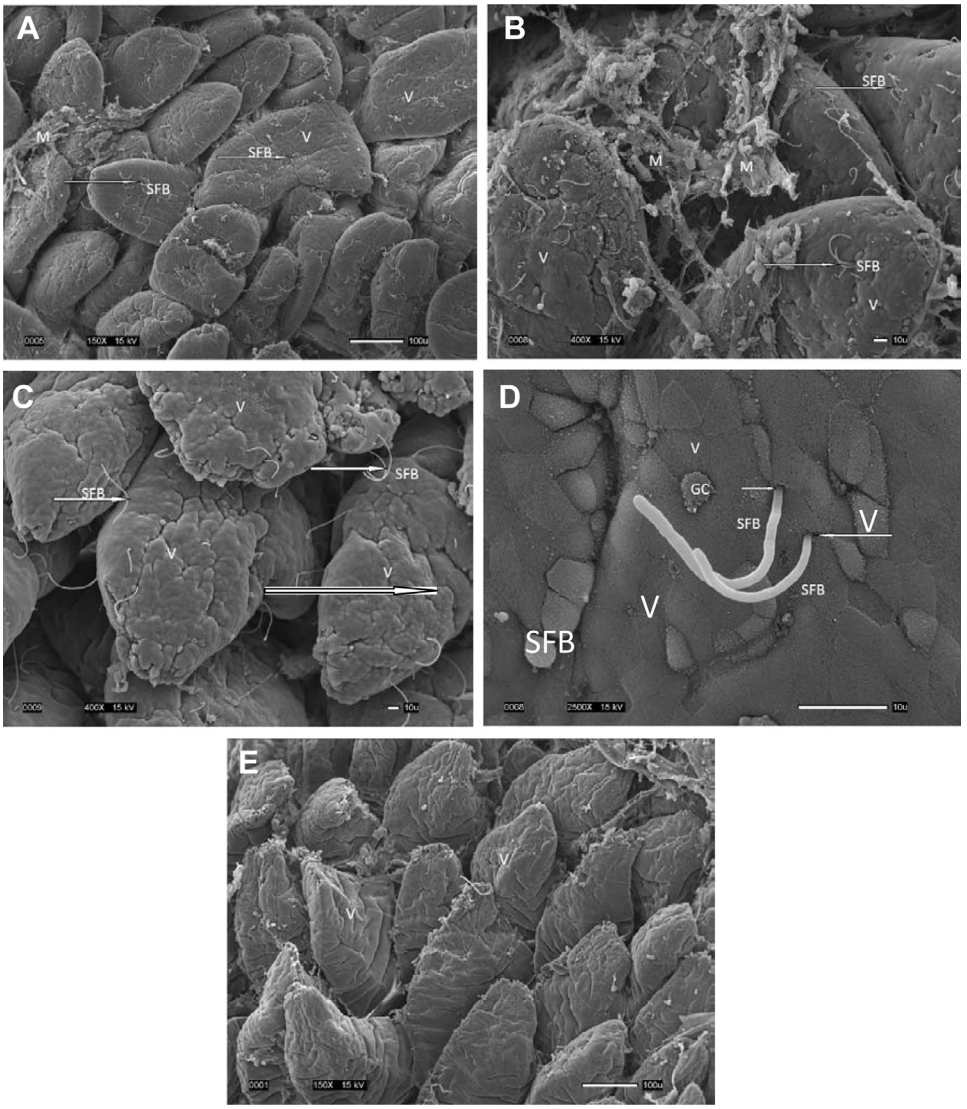


**Figure 6.** SEM images of SFB in the ileum of *Campylobacter*-challenged poults. The number of SFB (arrows) and amount of mucus secretion in DFM- (A, B, C) and MOS- (D) fed birds were higher than those in *Campylobacter*-challenged but not DFM and MOS–supplemented control birds (E). SFB attached to host cell distributing on villous (V) tip (arrow) (D). DFM, direct-fed microbial; MOS, mannan oligosaccharide; SEM, scanning electron microscopy; SFB, segmented filamentous bacteria.

1200EX transmission electron microscope (JEOL U.S.A.).

## RESULTS AND DISCUSSION

### *Scanning Electron Microscopy*

The villi of most specimens were mostly free of debris, because the intestinal tissues were prepared through gentle washing steps. Overall, the scanning electron micrographs of ileal samples showed more villi (not statistically significant, Table 2), and more mucus secretion (Figure 1E, F) with a high number of SFB (Figure 2A, B, D) in DFM and MOS supplemented groups, compared to the non-supplemented control group (Figures: 1D and 2E). In Salmonella challenged groups, the poults supplemented with DFM and MOS showed more mucus secretion (Figure 3A, B, E) and intact villi compared to non-supplemented group (Figure 3D). Figure 4B shows slight destruction of enterocytes on tips of villi in Salmonella challenged and non-supplemented control poults. The number of SFB in DFM treated birds (Figure 4A, C) was higher than non-supplemented group (Figure 4B). The C. jejuni infected group had distorted villi, and the enterocytes were lost, exposing the lamina propria (Figure 5F). In Campylobacter challenged birds, the number of SFB and amount of mucus secretion were higher in DFM and MOS supplemented groups (Figure 6A–D) compared to non-supplemented control group (Figure 6E).

The enteric morphology evaluation confirmed better villous integrity in the ileum of the probiotic and prebiotic treated poults compared with the control birds, indicating protection of the intestinal mucosa provided by DFM and MOS against the injury caused by the Salmonella and Campylobacter challenge. Therefore, based on these results, the DFM and MOS fed birds potentially made better use of nutrients, which also ensured

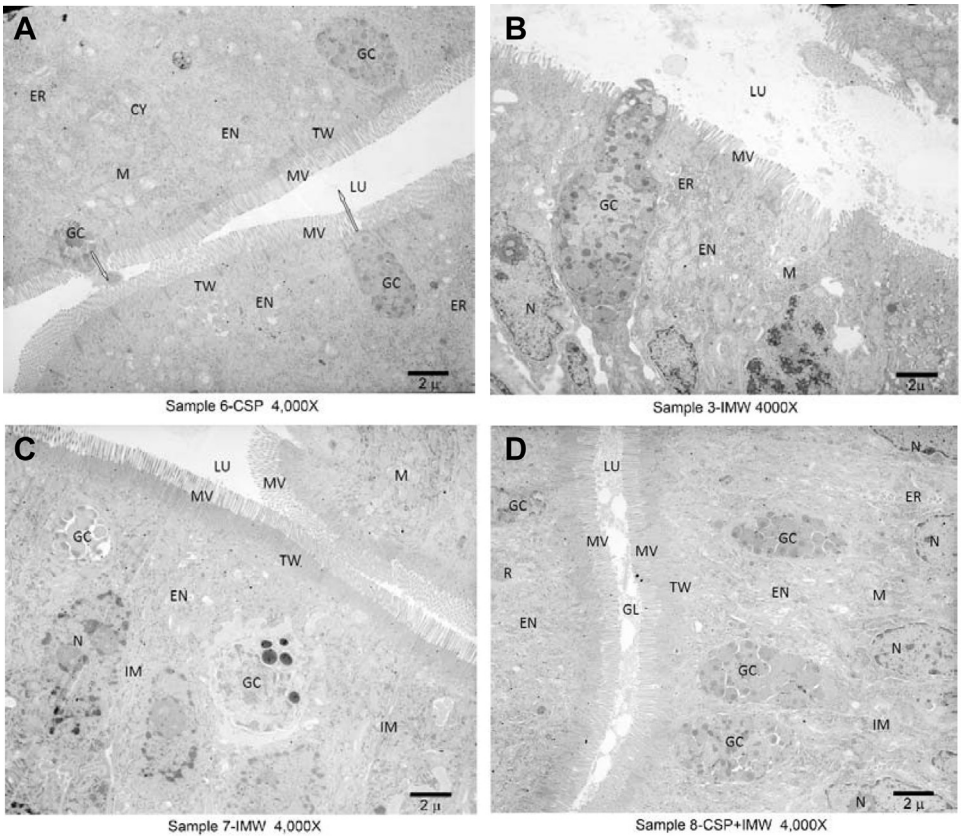


**Figure 7.** TEM images of the ileum of nonchallenged poults. The groups supplemented with DFM and MOS show intact microvillus architecture. The number of goblet cells (GC) in DFM- (A), MOS- (B, C) and mixture of DFM and MOS– (D) fed birds were high. In (D), upper parts of the enterocytes (EN) facing the lumen (LU) of the bowel show normal structure of the brush border (MV), the cytoplasmic organelles, and nucleus (N). In (A), note the opening of the goblet cell to the lumen of the intestine (arrow). (B) shows the normal structure of the cytoplasmic organelles such as mitochondria (M), cytoplasmic cisternae (CY), and endoplasmic reticulum (ER). The GC contained large numbers of secretory organelles lined by smooth membranes and containing dark-colored granular material. In photomicrographs (C) and (D), note the GC with their secretory granules and intercellular membranes (IM). DFM, direct-fed microbial; MOS, mannan oligosaccharide; TEM, transmission electron microscopy.

adequate structural preservation of the mucosal cells of the digestive tract. Better balance of the processes that determine enteric mucosal turnover resulted in better absorption and digestion. Increased amounts of mucus were observed in electron micrographs of DFM and MOS supplemented poults compared to control poults. Increased mucus in the gastrointestinal tract acts as a barrier as well as providing lubrication in the lumen to remove some pathogenic microorganisms.

These results are in agreement with reports by Gunal et al. (2006) and Chichlowski et al. (2007) where there was an increase in mucin glycoprotein concentration and number of goblet cells in the intestine of broilers fed a probiotic-supplemented diet. Caballero-Franco et al. (2007) reported a 60% increase in basal luminal mucin content with a probiotic treatment.

In many reports DFM reduce colonization and shedding of Salmonella and Campylobacter in poultry (Morishita et al., 1997; Johannsen et al., 2004). It has been reported that supplementation of broiler feed with yeast cell wall (Saccharomyces cervisiae, IMW50) improved intestinal integrity of the birds challenged with Salmonella Entritidis (Beirao et al., 2019). This is in agreement with findings herein. Johnson et al. (2017) reported a low level of colonization of C. jejuni in the upper intestinal tract and a high level of colonization in the ceca of broiler chickens. Johnson et al. (2017) reported that there was no significant difference between Campylobacter infected and uninfected birds in histopathological examination of cecal and intestinal sections. It has been predicted that decreasing Campylobacter colonization of poultry by 2-log10 will reduce human infections by 30-fold (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5362611/ Rosenquist et al., 2003). According to the USDA, FDA and CDC, 90% of Salmonella illness are attributed to sources other than chicken. Recently, the National Chicken Council (Anonymous, 2019) reported that the most recent government data indicates that: "98.5% of tests for Salmonella are negative for whole chickens at large plants; chicken producers have reduced Salmonella on whole chickens 66% over the past five years; since FSIS began testing chicken for Campylobacter in 2011, the industry has reduced the incidence by 30 percent; and Americans on average eat about 160 million servings of chicken every day, almost all of them eaten safely". The Food Safety and Inspection Service (FSIS) is the public health agency in United States Department of Agriculture (USDA) that

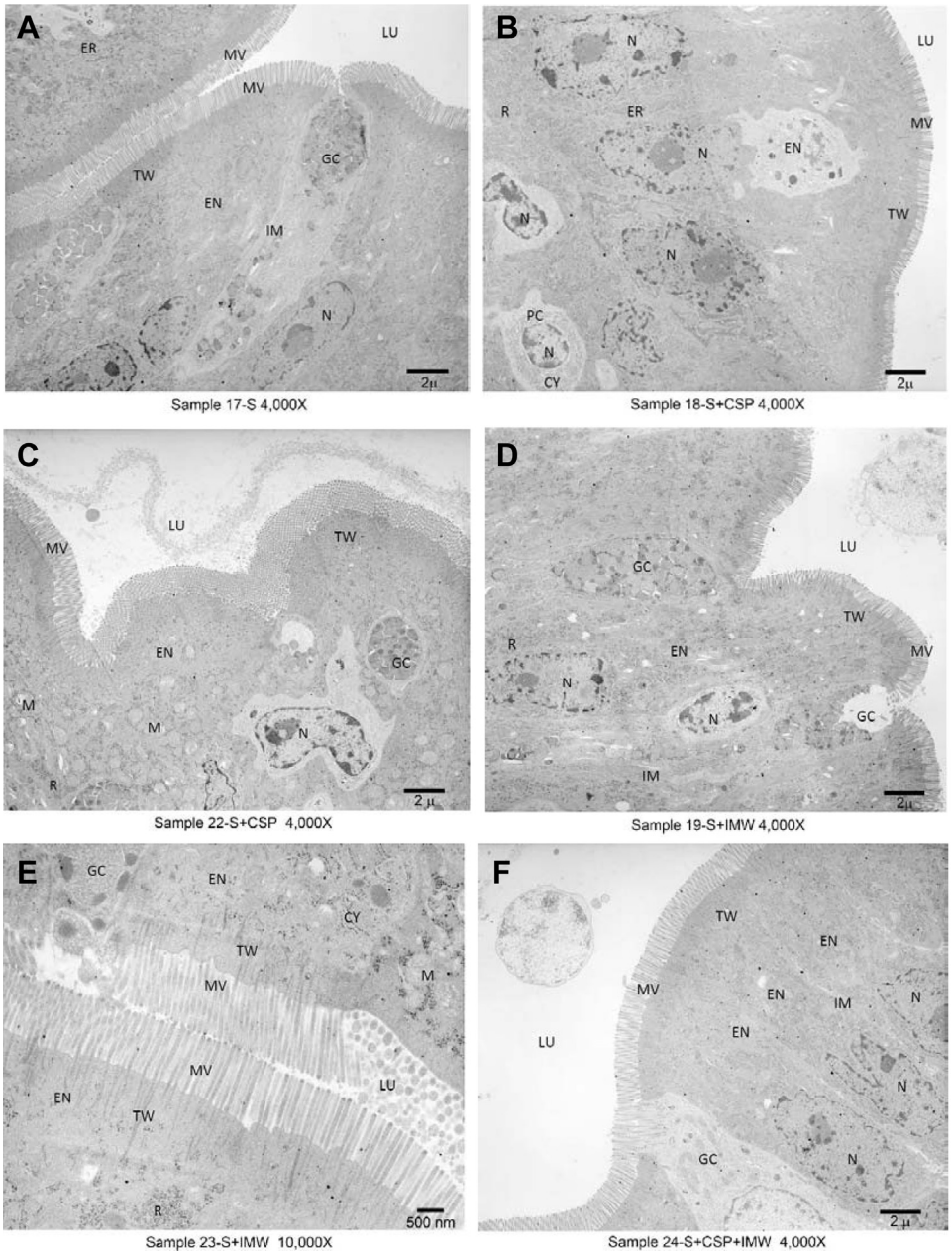


**Figure 8.** TEM images of the ileum of *Salmonella*-challenged poults. The number of goblet cells (GC) in DFM- (B, C), MOS- (D, E), and mixture of DFM and MOS– (F) supplemented birds was high. Note GC opening to the lumen (LU) of the bowel in *Salmonella*-challenged but not DFM and MOS– supplemented birds (A) and the normal structure of cytoplasmic organelles, nucleus (N) of enterocytes (EN), and plasma cell (PC) in (B). (C) shows a GC and normal structure of the cytoplasmic organelles such as mitochondria (M), microvilli (MV), and nuclei (N) of enterocytes (EN) facing the LU of the bowel. (D) presents GC opening to the LU of the intestine. The photomicrograph (E) presents facing brush border (MV) of the enterocytes (EN) to the LU of the bowel. A GC opening to the LU, presence of mitochondria (M), and cytoplasmic cisternae (CY) in enterocytes (EN) can be seen in this picture. (F) shows an opening GC to the lumen and presence of enterocytes (EN) with normal MV and mitochondria (M). Several enterocytes and 1 GC are facing toward the LU of the bowel (F). Notice that the GC is well covered by MV. During discharge of the granular material from the cell, the MV separate and the material escapes into the LU in the form of mucous droplets. DFM, direct-fed microbial; MOS, mannan oligosaccharide; TEM, transmission electron microscopy.

is responsible for inspection at broiler chicken processing facilities (those facilities that process chickens for meat). The U.S. meat and poultry inspection system complements industry efforts to ensure that the nation's commercial supply of meat and poultry products is safe, wholesome and correctly labeled and packaged. For consumers, the bottom line is that chicken is safe when properly cooked and handled, and that chicken producers and processors are continually working to make them even safer. Instructions for safe handling and cooking are printed on every package of meat and poultry sold in the United States – when followed, one can be assured of a safe eating experience every time.

## Transmission Electron Microscopy

The transmission electron microscope images show more goblet cells and intact microvilli architecture in DFM and MOS supplemented groups (Figure 7). In this electronmicrograph, nuclei in the villous epithelial

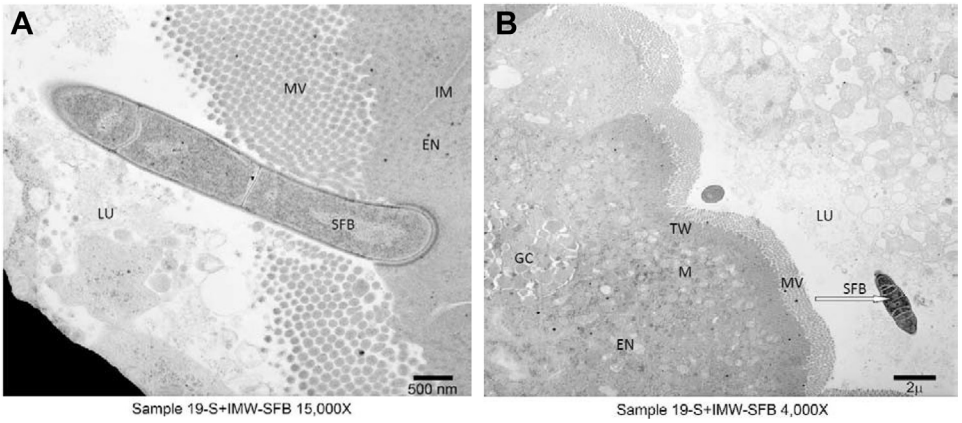


**Figure 9.** TEM images of SFB in the ileum of *Salmonella*-challenged prebiotic-fed poults. Image (A) shows an epithelial cell containing an SFB embedded in an enterocyte (A, EN) (magnification, 15,000×). The brush border membrane (MV) of the epithelial cell is unaffected. In image (B), the SFB is sectioned tangentially, includes intracellular bodies (arrow) and appears to be floating in the intestinal lumen (LU) (magnification, 4000×). In this picture, a goblet cell (GC) and an enterocyte (EN) with normal microvilli (MV) and mitochondria (M) are facing the lumen (LU) of the ileum. SFB, segmented filamentous bacteria; TEM, transmission electron microscopy.

cells of the ileum were located centrally and the mitochondria were evenly distributed. In photomicrographs 7 C and D, note the goblet cells (GC) with their secretory granules, and intercellular membranes (IM). The endoplasmic reticulum and other cytoplasmic organelles were present and appeared to have a normal morphology. Few mitochondria are seen in intimate relation to rough endoplasmic reticulum (Figure 7). The goblet cells containing numerous mucus granules are placed between the villous epithelial cells and secrets mucus (the protective layer of mucopolysaccharides) into the lumen of the intestine (Figure 7). Several mitochondria (M) are seen in the cytoplasm of the enterocytes (Figure 7). In *Salmonella* challenged birds, the goblet cells containing numerous mucus granules were seen between the villous epithelial cell, and mucus was on the luminal surface of enterocytes (Figure 8). More mucus secretion is shown in DFM and MOS supplemented groups (Figure 8C, D). Several enterocytes and one goblet cell (GC) are facing toward the lumen of the bowel (Figure 8F). Notice that the goblet cell is well covered by microvilli (MV). During discharge of the granular material from the cell, the microvilli separate and the material escapes into the lumen (LU) in the form of mucus droplets.

The brush border consisted of individual cylindrical microvilli. This network is called the terminal web (Figure 8). The lateral membranes of adjacent enterocytes are held together by various junctions. The tight junction (zonula occludens) is situated immediately bellow the microvilli and is formed by the fusion of the outer leaflets of the plasma membrane, thus sealing the intercellular spaces from the outer environment, i.e. the luminal space. The intermediate junction (zonula adherens) lies just below the tight junction and appears as an electron-dense small linear area separated by a small gap and surrounded by fuzz extending into the adjacent cytoplasm. The third most prominent and numerous lateral cell junctions are the desmosomes (macula adherens). These are dense plaques composed of four membrane leaflets and a gap surrounded by dense cytoplasmic fuzz (Figure 8). At high magnification of a longitudinal section of microvilli (Figure 8E) one can note that microvilli are coated by glycocalyx. Glycocalyx, as well as many enzymes localized within the striated border, participate in the cell's absorptive processes (Denbow, 2015). The fine filaments of microvilli also extend from the sides and tips of the free surface of their membrane into the lumen of the bowel to form part of the glycocalyx.

Electron microscopic examination revealed that the filamentous organisms penetrated absorptive enterocytes (Figure 9). In this figure, the attached end of SFB appeared to embed between the microvilli that were pushed aside and sometimes disappeared in the depth where goblet cells had opened.

Several enterocytes (EN) are seen facing the lumen of the bowel (LU) (Figure 10). The apical plasmalemma is broken and the disrupted cell contents have been released to the lumen of the intestine (Figure, 10F). This photomicrograph shows a rupture of the brush border and extrusion of the cell contents toward the lumen of the intestine. Endocrine cells containing dense secretory granules in the cytoplasm and oval nuclei (N) are also seen (Figure 10). Various cell types such as enterocytes, goblet cells and cells of connective tissue plus fine strands of collagen are seen in the lamina propria of intestine of MOS supplemented bird (Figure 10C, E). In goblet cells (GC), large numbers of secretory organelles lined by smooth membrane and containing dark-colored granules material are seen. Each microvillus was seen as an extension of the apical plasmalemma and as a continuous membrane enveloping a structural complex, the cytoskeleton (Figure 10). The core of the microvilli consist of filaments which are arranged in an axial fashion. The filaments extend to the apical cytoplasm of the enterocyte where they join those from neighboring microvilli in a network of interlacing fibers arranged parallel to the surface of the cell.

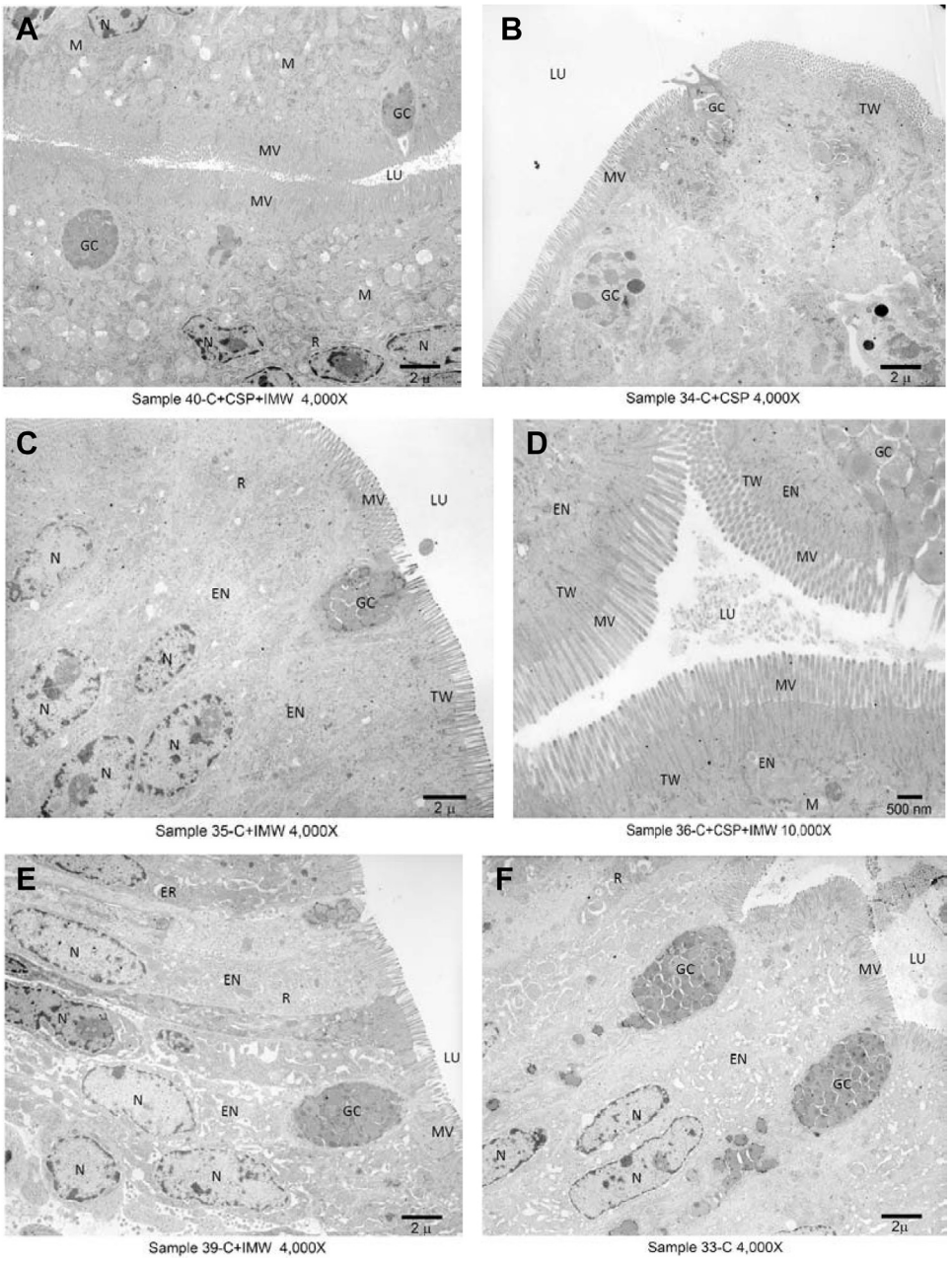


**Figure 10.** TEM images of the ileum of *Campylobacter*-challenged poults. Goblet cells (GC) opening to the lumen (LU) of the intestine and enterocytes (EN) can be seen in DFM- (B), MOS- (C, E) and the mixture of DFM and MOS– (A, D) supplemented birds and nonmedicated, *Campylobacter*-challenged birds (F). Fine structural of ileal epithelial cells and aggregated mitochondria (M) are present in most of the figures. In figure (C), longitudinal sections of several microvilli (MV) and terminal web (TW) project from the apical portion of the enterocyte cytoplasm (EN) into the LU of the bowel. Figure (F) shows longitudinal sections of EN and 2 goblet cells (GC). Notice that the GC are well covered by MV. During discharge of the granular material from the cells, the MV separate and the material escapes into the LU in the form of mucus droplets. The apical plasmalemma is broken, and the disrupted cell contents are being released to the lumen of the intestine (F). This photomicrograph shows a rupture of the brush border and extrusion of the cell contents toward the lumen of the intestine. Endocrine cells with dense secretory granules in the cytoplasm and oval nuclei (N) are seen. DFM, direct-fed microbial; MOS, mannan oligosaccharide; TEM, transmission electron microscopy.

The pathological findings in the present study such as atrophy and destruction of villi in *Salmonella* and *Campylobacter* infected birds confirm the pathogenicity of these organisms to the turkey poults. It has been reported that alteration of villi and microvilli decrease specific functional enzymes in the intestinal epithelial cells causing significant effects on nutrient absorption. The importance of the villi and microvilli in digestion and absorption of nutrients has been described by Denbow (2015).

Denbow stated that the brush border is a digestive-absorptive surface organelle less complex than mitochondria, but a structurally integrated subcellular organelle, controlling and interacting with the internal environment of the enterocytes as well as forming its luminal surface. Enzymes catalyzing digestive and absorptive functions seem to be spatially arranged in the brush border so as to offer a kinetic advantage for absorption, not only for digestive feedstuffs, but also for sodium, and possibly other ions by a mechanism of

cooperative interaction between these and other absorbed molecules in the mobile carrier transport system (Denbow, 2015). In addition, one could speculate that decrease of feed consumption in animals infected with pathogenic microorganisms such as *Salmonella* (Rahimi et al., 2009) and *Campylobacter* (Ebrahimi et al., 2016) could arise from damage to the intestinal mucosa that signals the neural center (hypothalamus) of the brain. A decrease in the intestine's ability to take up specific nutrients, because of the lesions caused by pathogenic microorganisms, may play a role in the debilitation and wasting that often follows exposure to pathogens. Extrapolating this concept further, one would include the wasting effect caused by other pathogens to be a result of intestinal lesions such as those seen in this study.

The mitochondria carry out most cellular oxidation and produce the bulk of the cell's ATP. The matrix space contains a large number of different enzymes (Bottje, 2015). The mitochondria are important cell organelles, which are affected by many toxic materials. The swelling and disruption of mitochondria in the enterocytes of chickens fed polychlorobiphenyl is reported by Rahimi (2002).

Welkos (1984), in a study on susceptibility of chicks to *C. jejuni,* reported that three-day-old chicks did not develop enteritis after oral inoculation, but chicks infected within 12 h of hatching did develop gastroenteritis. *C. iejuni* was recovered throughout the intestine; the highest concentrations were present in the caecum and large intestine. Organisms resembling *C. jejuni* were seen within the intestinal epithelium and lamina propria by electron microscopy. Sokale et al. (2019) reported that supplementation of diet with *Bacillus subtilis* could improve production performance of chickens by modulating the composition of the intestinal microflora, which is in agreement with the findings herein.

### Segmented Filamentous Bacteria

The SFB were seen in the transmission electron micrographs attached to the apical membrane of epithelial cells. The proximal bacterial segment attached to and penetrated the apical cell membrane. The epithelial cell closely paralleled the shape of the bacteria and contained an adjacent line of intracellular electron-dense material (Figure 9).

The SFB, which are *Clostridia*-related spore-forming, gram-positive bacteria, are well-known members of these nonculturable populations. These bacteria are well known for their unique morphology and tight attachment to intestinal epithelial cells and Peyer's patches, which strongly suggest their involvement in the maturation of the gut immune system (Klassen et al., 1993; Umesaki et al., 1995). It has been reported that SFB have an important role in inducing immune response (Meyerholz et al., 2002) and in exerting a potential antagonistic effect against pathogenic microorganisms in the GI tract (Heczko et al., 2000). Within the follicle-associated epithelium, membranous cells are involved in the continuous sampling of antigens from the lumen. In fact, it was found that SFB colonization enhances the luminal IgA production. These bacteria are sufficient to induce the appearance of CD4 (+) T helper cells that produce interleukin-17 and interleukin-22 (TH 17 cells) in the lamina propria (Suzuki et al., 2004). SFB are autochthonous bacteria colonizing the ileum of many young animals by attaching to intestinal epithelial cells. These nonpathogenic bacteria strongly stimulate the mucosal immune system and induce intestinal epithelial cells to express major histocompatibility complex class II molecules (Yamauchi and Snel, 2000). The SFB might increase the resistance of the host to infectious diseases (Garland et al., 1982).

The SFB may help to consolidate the immune system, and they may also prevent colonization of pathogens in a competitive and mechanical way (Desvaux, 1995). The SFB may affect host resistance to a variety of enteric pathogens. The core of the microvilli consists of filaments, which are arranged in an axial fashion. The filaments extend to the apical cytoplasm of the enterocyte where they join those from neighboring microvilli in a network of interlacing fibers arranged parallel to the surface of the cell. This network is called the terminal web. The SFB specifically induce the differentiation of effecter Th 17 cells in the lamina propria (Klassen et al., 1992; Yamauchi and Snel, 2000).

## CONCLUSION

The observation of ultrastructural changes of intestinal mucosa due to infection with *Salmonella* and *Campylobacter* as affected by diet was the main purpose of this study. The electron microscopic assessment of intestinal mucosal cells from the *Salmonella*- and *Campylobacter*-infected poults in this experiment demonstrated degenerative changes in the intestinal mucosa. Therefore, pathogenic bacterial infection of the intestinal mucosa is directly responsible for the development of malabsorption and malnutrition processes and likely contributes to changes in the normal gut flora, thus further adding to loss of gut health. The dietary DFM and MOS used in this study appeared to confer intestinal health benefits to poults by improving their morphological development. There is potential for DFM and MOS to prevent and control pathogenic microorganisms, such as *Salmonella* and *Campylobacter*, from affecting the intestinal morphology integrity of turkey poults. Numbers of SFB (which affect the maturation of the gut immune system in birds) were highly increased in the ileum of the DFM and MOS–supplemented groups compared with control-fed poults.

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